Inducible Innate Resistance of Lung Epithelium to Infection

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Abstract

Most studies of innate immunity have focused on leukocytes such as neutrophils, macrophages, and natural killer cells. However, epithelial cells play key roles in innate defenses that include providing a mechanical barrier to microbial entry, signaling to leukocytes, and directly killing pathogens. Importantly, all these defenses are highly inducible in response to the sensing of microbial and host products. In healthy lungs, the level of innate immune epithelial function is low at baseline. This is indicated by low levels of spontaneous microbial killing and cytokine release, reflecting low constitutive stimulation in the nearly sterile lower respiratory tract when mucociliary clearance mechanisms are functioning effectively. This contrasts with the colon, where bacteria are continuously present and epithelial cells are constitutively activated. Although the surface area of the lungs presents a large target for microbial invasion, activated lung epithelial cells that are closely apposed to deposited pathogens are ideally positioned for microbial killing.

INTRODUCTION

Microbes entering the bodies of multicellular eukaryotes must first cross an epithelial cell layer. Besides functioning as physical barriers to prevent infection, mammalian epithelial cells are able to sense the presence of microbes and to respond by augmenting their barrier function, signaling to leukocytes, and directly killing pathogens. Although signaling to leukocytes has received considerable attention, augmented barrier function and pathogen killing have received less. Conditional pathogen killing by epithelial cells, in particular, is an important aspect of innate resistance to infection that merits further attention in understanding the homeostasis of epithelial surfaces throughout the body and in manipulating innate immunity therapeutically.

It should not come as a surprise that mammalian epithelial cells are capable of highly inducible antimicrobial defenses because innate immune function was first elucidated in insects in which the systemic response to infection is mediated by the release of antimicrobial peptides from epithelial cells of the fat body into the hemolymph (1, 2). However, leukocytes that are specialized for immune functions have traditionally dominated the attention of mammalian immunologists. Furthermore, the initial identification of Toll-like receptor (TLR) signaling in mammalian biology focused on costimulatory molecules required for adaptive immune responses (3), reinforcing the focus on leukocytes. Although the abilities of professional immune cells to detect and kill pathogens are impressive, nonspecialist epithelial cells have retained such capabilities through evolution. This observation suggests that, rather than replacing the innate immune function of epithelial cells in higher eukaryotes, leukocytes complement these functions to collaborate in host defense.

Resistance: the strategy of host survival of infection that is associated with a reduction in pathogen burden; this is contrasted with tolerance, the strategy of generating a host phenotype indifferent to the pathogen burden

Innate immunity: host antimicrobial defenses involving detection of conserved microbial molecular motifs by host germline-encoded pattern recognition receptors and characterized by rapid but transient responses of both leukocytes and parenchymal cells

Hemolymph: fluid in the body cavity of insects, homologous to vertebrate blood, with most proteins produced by the fat body, homologous to the vertebrate liver, with functions in both metabolism and immunity

TLR: Toll-like receptor

Adaptive immunity: host antimicrobial defenses involving detection of specific pathogen antigens by somatically recombined receptors and characterized by clonal expansion of pathogen-specific lymphocytes and immunologic memory

INDUCIBLE INNATE RESISTANCE OF LUNG EPITHELIUM

Several lines of evidence have pointed to the inducibility of innate immune defenses in lung epithelial cells. First, the epithelial cells of lower eukaryotes show robust inducible defenses, as discussed above, and the barrier epithelia of *Drosophila*, including those of the trachea, Malpighian tubules, gut, and reproductive tract, show specific patterns of antimicrobial peptide expression (1, 2). Second, cultured lung epithelial cells show the inducible expression of antimicrobial polypeptides in vitro, and transgenic overexpression of some of these has been shown to result in increased resistance to infection in vivo (4–8). Third, lung epithelial cells show remarkable structural and molecular plasticity during inflammation, suggesting that lung epithelial defensive functions are similarly plastic (9). Fourth, modest increases in resistance to bacterial infection by the airway route have been found after exposure of the lungs to single innate immune ligands such as endotoxin (10–18), although the role of the epithelium was not isolated experimentally.

To strongly induce lung defenses in vivo, we exposed mice to an aerosolized lysate of the bacterium nontypeable *Haemophilus influenzae* (NTHi), reasoning that this would stimulate the epithelium with a complex mixture of pathogen-associated molecular patterns (PAMPs) in proportions that reflect a natural exposure. This stimulation rapidly resulted in a high level of resistance to a broad array of microbial pathogens (9, 19, 20). In the initial studies (19), resistance to *Streptococcus pneumoniae* reached a maximum 4 h after stimulation, remained at the maximal level for 24 h, then gradually declined over several days (**Figure 1*a***). Host protection was mirrored by augmented microbial killing within the lungs (**Figure 1*b***). In subsequent studies, the NTHi lysate was shown to induce resistance to all pathogens tested, including Gram-positive and Gram-negative bacteria, the spore-forming NIAID class A bioterror agent *Bacillus anthracis*, the fungus *Aspergillus fumigatus*, and influenza virus (9, 20). In each case, increased host survival was associated with a reduction of lung pathogen burden, indicating that host protection occurs through a resistance mechanism.

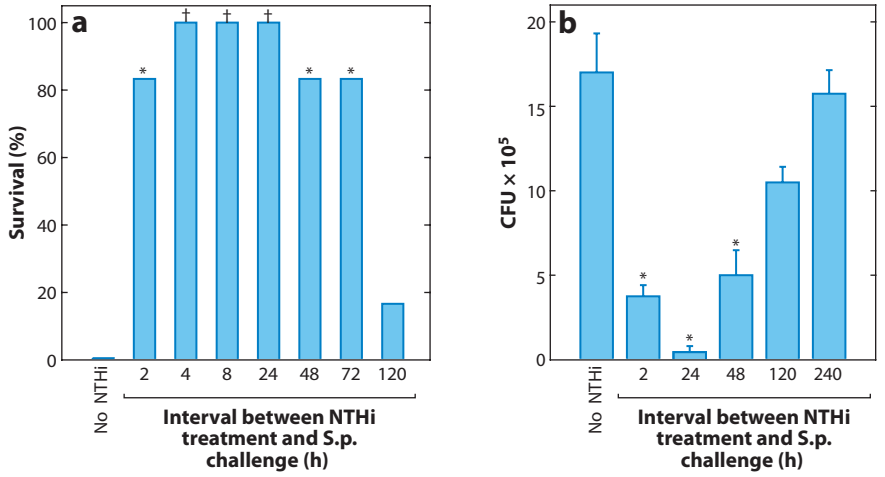

Figure 1

Time course of induced innate resistance in the lungs. (*a*) Host survival. Mice were pretreated in groups of six with an aerosolized lysate of the bacterium nontypeable *Haemophilus influenzae* (NTHi) to stimulate innate immunity, then challenged as a single group with live aerosolized *Streptococcus pneumoniae* (S.p.) ($6.1–10^{10}$ CFU ml^{-1} for 60 min). Survival at 7 days is shown as a function of the interval between treatment and challenge ($^{*}p = 0.015$, $^{\dagger}p = 0.002$, treated versus untreated). (*b*) Bacterial counts in the lungs. Mice were pretreated in groups of four with an aerosolized NTHi lysate at various time points, then challenged as a single group with live aerosolized S.p. ($2.1–10^{10}$ CFU ml^{-1} for 60 min). Lungs were removed immediately after the aerosol challenge, homogenized, and plated for bacterial culture (mean ± SEM, $^{*}p < 0.05$ for treated versus untreated). From Reference 19 with permission.

The following evidence supports the dominant role of the epithelium in stimulated innate resistance of the lungs. First, resistance is local, such that there is no protection against an intraperitoneal or intravenous microbial challenge after aerosol stimulation of the lungs (19). Second, resistance can be induced in mice deficient in neutrophils, macrophages, or mast cells (19), as well as in mice deficient in dendritic cells, natural killer cells, or lymphocytes (S.E. Evans, unpublished data). Thus, neither resident nor recruited leukocytes are required for stimulated innate resistance of the lungs, although it is likely that resident leukocytes amplify the sensing of PAMPs by signaling to epithelial cells locally (see below), and neutrophil recruitment is clearly important for clearing large microbial inocula in mice and in the susceptibility to infection of neutropenic patients (19, 21, 22). Third, important roles for innate immune signaling in lung parenchymal cells have been demonstrated in the control of bacterial and viral infections (23–26). Fourth, efficient direct microbial killing by epithelial cells stimulated with PAMPs or cytokines in vitro has been demonstrated (9, 21, 27), in contrast to dendritic cells and macrophages stimulated in vitro that do not kill bacteria efficiently (9). Fifth, consistent with the functional capability of isolated epithelial cells to kill pathogens, multiple epithelium-derived antimicrobial proteins are found upregulated in lung lining fluid by proteomic analysis after stimulation with microbial products (19). Similarly, multiple antimicrobial proteins are upregulated by gene expression microarray in leukodepleted lungs and isolated epithelial cells after stimulation with microbial products or cytokines (9, 21), although identification of the critical epithelial effector mechanisms has not yet been determined (see below, Epithelial Effector Mechanisms of Inducible Resistance).

Together, these results indicate that respiratory epithelial cells are capable both of sensing

Nontypeable *Haemophilus influenzae* (NTHi): an unencapsulated (hence nontypeable) strain of a Gram-negative bacterial pathogen that is frequently cultured from the lungs of patients with chronic respiratory disease

Pathogen-associated molecular pattern (PAMP): stereotypic molecular motifs conserved across microbial species that are recognized by pattern recognition receptors, triggering innate immune responses

innate stimuli and of carrying out effector responses that underlie inducible innate resistance. In *Hydra*, a simple metazoan that lacks mobile phagocytes or hemolymph, epithelial cells sense microbial infection by a leucine-rich repeat (LRR) receptor and respond by synthesizing and secreting antimicrobial polypeptides (28). In plants that are even more distantly related to mammals, LRR receptors similarly recognize PAMPs and initiate complex and vigorous resistance responses (29). This conservation indicates that epithelial activation is an ancient system of defense in multicellular eukaryotes. In mammals, in which a complex array of leukocytes collaborate in host defense, epithelial cells engage in bidirectional signaling with leukocytes while retaining the ability to mount a vigorous defense of their own.

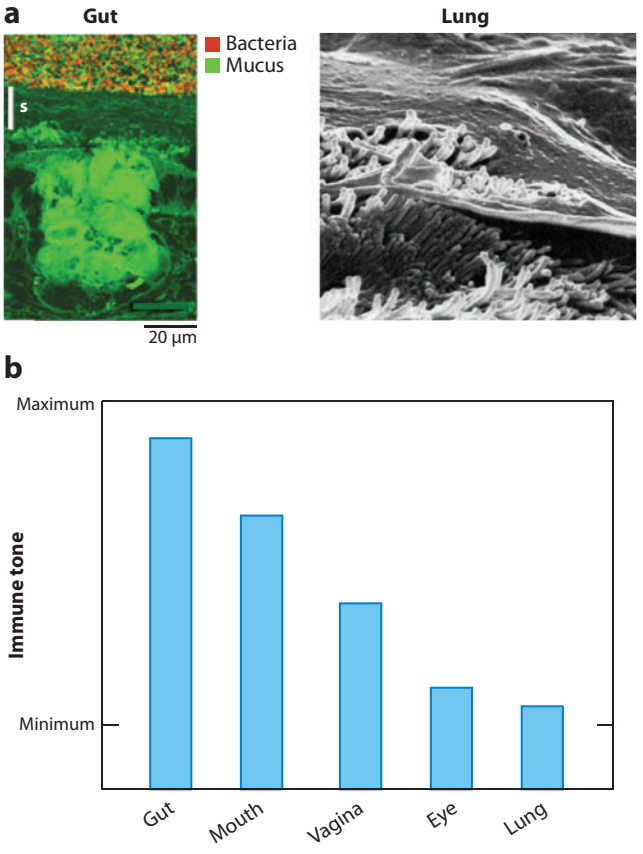

Figure 2

Architecture and innate immune tone of mucosal surfaces. (*a*) Architecture of colon and lung. The architecture of the mouse colon is illustrated on the left, showing the epithelium with intracellular mucin (*intense green*), extracellular mucin (*faint green*), and overlying bacteria (*red*). The mucus gel layer is thick (up to 500 μm), is adherent to the epithelium, and is composed of a loose outer layer that contains bacteria and a firm stratified (s) inner layer that is essentially devoid of bacteria. From Reference 30 with permission. Scale bar is 20 μm. The architecture of the airway is illustrated on the right, showing a thin mucus layer (5 μm in distal airways to 50 μm in proximal airways) overlying a periciliary liquid layer ~7 μm in depth. Aspirated microbes become entrapped in the mucus gel layer and are rapidly swept out of the lungs by ciliary action, keeping the lungs nearly sterile. (*b*) Baseline innate immune tone of mucosal surfaces. The epithelial resistance of mucosal surfaces of the mammalian body is postulated to be related to exposure to microbial products. The tone of the lung and gut is illustrated in relative terms with regard to each other and to the minimal and maximal points of the scale; the tone of all other surfaces is speculative.

ARCHITECTURE OF EPITHELIAL SURFACES

To understand the innate immune activation of epithelial cells, it is important to appreciate their microenvironments. Epithelial surfaces throughout the mammalian body are in constant or episodic contact with microbial pathogens. A variety of physical strategies are utilized to prevent microbial penetration of epithelial layers that would result in deep tissue or bloodstream infection. For example, the skin utilizes an impermeant outer layer of dead keratinized cells. The stomach utilizes acid that makes its lumen inhospitable to microbes and that renders the proximal small bowel and interconnected pancreatic and biliary trees nearly sterile. The distal small bowel and the large bowel, however, are in constant proximity to a rich microbial flora (**Figure 2*a***), albeit separated by a thick mucus gel layer (30, 31). The lungs utilize a strategy of directed airflow to induce impaction and sedimentation, together with mucociliary and cough clearance, to rapidly remove aspirated microbes from the lower respiratory tract (32–34). The importance of mucociliary clearance in the lungs is indicated by the chronic inflammation that occurs in human subjects with ciliary dyskinesia (33, 35) and the progressive lethal lung inflammation that occurs in mice lacking the gel-forming mucin Muc5b (C.M. Evans, personal communication). Other mucosal surfaces such

as the mouth, eyes, and urinary tract employ both overlapping and additional strategies.

These distinct epithelial microenvironments with markedly different baseline exposure to microbial products reveal differences in the baseline induction of innate immune defenses, with the most informative contrast being between the lungs, which are nearly sterile, and the distal bowel, which is heavily colonized with microbes (**Figure 2*b***). In the lungs, innate immune activation (tone) is low, as indicated by the low level of microbial killing within the lungs of mice at baseline (9, 19, 20) or by unstimulated lung epithelial cells in vitro (9, 21). From this low baseline, innate resistance is highly inducible (**Figure 2**). In the distal bowel, in contrast, innate immune tone is substantial at baseline, as indicated by rapid bacterial killing within an isolated ileal loop (36). Bacterial killing can be reduced by prior exposure of mice to antibiotics that reduce commensal microbes, and can be restored by exposure to TLR agonists. Thus, diverse epithelial cells appear to be capable of a range of innate immune tone determined by local microbial signals and modulated by innate and adaptive immune cells (37). Consistent with the ability of epithelial cells to maintain tone, we have found no tachyphylaxis of resistance despite repetitive stimulation of the lungs (9, 20), similar to the tachyphylaxis of inflammation, but not of resistance described by others (38).

EPITHELIAL SENSING OF INNATE STIMULI

For the induction of microbial resistance, lung epithelial cells must sense stimuli directly from microbes or indirectly from nonepithelial host cells and extracellular molecules. Evidence exists that lung epithelial cells are highly responsive to both categories, with the relative importance of individual stimuli in inducing resistance in vivo against specific pathogens a subject of ongoing study.

Direct Sensing of Microbial Products

Jawed vertebrates have two distinct means of detecting the presence of pathogens, innate and adaptive immunity, distinguished fundamentally by the nature of their receptors. Adaptive immune recognition relies upon antigen receptors expressed by T and B lymphocytes that are encoded by somatically recombined gene segments, resulting in an immense library of receptors for precise epitopes. The clonal distribution of highly specific antigen receptors allows for expansion of pathogen-appropriate lymphocyte populations and for immunologic memory but limits the number of pathogens that can be detected by individual cells and requires prior exposure to the pathogen (39). In contrast to the highly refined epitope sensing of adaptive immune receptors, innate immune receptors rely on recognition of conserved molecular features common to multiple microorganisms (PAMPs). Germline-encoded pattern recognition receptors (PRRs) bind PAMPs, allowing recognition of a large number of different microorganisms, although the broad conservation of these structural motifs does not generally allow discernment of pathogenic from nonpathogenic microorganisms (40–44). PAMPs may be either surface-associated or internal elements of microbes and are best suited to inducing efficient innate responses when they are invariant across many species, critical to microbial metabolic or virulence processes, and are not present in host products (39). Additionally, some PRRs identify host molecules that are expressed in response to infection or host molecules that have been modified in the course of infection, known as danger signals or damage-associated molecular patterns (DAMPs). PRRs can be soluble, bound to cell surface or endosomal membranes, or cytosolic in distribution. Recognition of PAMPs and DAMPs by PRRs activates intracellular signaling cascades, leading to the expression of effector molecules involved in microbial defense, inflammation, and modulation of adaptive immunity (45). The past two decades have

Leucine-rich repeats (LRRs): common molecular sequences of many pattern recognition receptors that generally occur within pathogen recognition domains

Pattern recognition receptors (PRRs): membrane-associated, cytosolic or secreted host products that recognize conserved molecular patterns on pathogens, initiating innate immune responses. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), class A scavenger receptors (SR-As), and macrophage receptors with collagenous structure (MARCOs)

Damage-associated molecular pattern (DAMP): molecular motifs expressed on or released by infected or injured host cells, also known as danger signals or alarmins

witnessed the identification of several distinct classes of PRRs.

Toll-like receptors. TLRs were the first class of innate immune receptors identified and remain the best characterized. TLRs are highly conserved class I transmembrane proteins, consisting of an ectodomain with multiple LRRs for pattern recognition, a single membrane-spanning α-helix, and a Toll/interleukin-1 receptor (TIR) domain for intracellular signaling. The structure and function of TLRs are described in detail in a number of recent reviews (3, 43, 46, 47). Upon ligand binding, signal transduction occurs via receptor-specific recruitment of cytosolic TIR adaptor protein combinations. In concert with one or more of the four other adaptors, MyD88 is involved in more TLR signaling than any other TIR adaptor (42–44, 48, 49). The MyD88-independent signaling events observed from TLR3 and TLR4 utilize the TIR adaptor Trif, with or without TRAM (TRIF-related adaptor molecule) (43, 48, 49). Numerous recent reviews describe the current understanding of TLR downstream signaling (3, 43, 46, 47).

The primary ligand for TLR4 and its coreceptor CD14 is a complex of the soluble host protein MD2 with the lipid A moiety of bacterial lipopolysaccharide (LPS), allowing detection of Gram-negative pathogens. Many Gram-positive bacteria, parasites, and some Gram-negative bacteria can be recognized by TLR2-dependent binding of lipopeptides, such as peptidoglycan, lipotechoic acid, and atypical LPS. TLR2 generally functions as a heterodimer with TLR1 or TLR6, with TLR2/1 recognizing triacylated lipopeptides (e.g., Pam_3CSK4) and TLR2/6 recognizing diacylated lipopeptides (e.g., Pam_2CSK4, MALP2). Fungal zymosan is a ligand for TLR2/6, as well. A highly conserved motif of flagellin that spans many bacterial species is recognized with high affinity by a well-defined TLR5-binding site.

Four TLRs recognize microbial nucleic acids. TLR3 recognizes double-stranded RNA (dsRNA) and can be stimulated by synthetic mimetic copolymers, such as poly inosine:poly cytosine (poly I:C). TLRs 7 and 8 recognize U-rich (i.e., nonmammalian) single-stranded RNA (ssRNA) as well as imidazoquinolones such as imiquimod and resiquimod. TLR9 detects DNA with unmethylated CpG motifs, which differ from mammalian DNA, which is typically methylated. A number of host danger signals, such as heat shock proteins, are also protein ligands for TLRs (3, 43, 46, 47).

Experiments in TLR-deficient mice provide insight into the roles of TLR in defense against pneumonia. Mice spontaneously deficient in TLR4 show increased susceptibility to *H. influenzae* and *Escherichia coli* pneumonia associated with impaired pathogen clearance (50, 51). Dual deficiency in TLR4 and CD14 was also found to increase susceptibility to RSV (respiratory syncytial virus) (52), consistent with the observation that TLR4 is a coreceptor for RSV fusion protein. TLR5-deficient mice have increased susceptibility to lung pathogens including *Legionella pneumophila* (53). Interestingly, mice deficient in both TLR2 and TLR4 do not demonstrate hypersusceptibility to *Pseudomonas aeruginosa* (54), even though mutations of pseudomonal flagellin that prevent TLR5 binding result in impaired bacterial clearance and host survival (55). Surprisingly, TLR3 deficiency was reported to confer a survival advantage in an influenza virus pneumonia model (56), presumably through prevention of an excessive host response. However, the finding that intranasal pretreatment with TLR3 agonists protects against influenza pneumonia highlights the requirement for precise regulation of TLR-dependent responses in microbial defense (57).

It has been apparent for nearly a decade that microbial products may stimulate innate responses from the respiratory epithelium, and it has been suspected that TLRs contribute to those responses since they were first identified in mammals (4). Although there are 13 known TLRs in humans, most is known about TLRs 1–9 and their murine orthologs (3, 47). Polymerase chain reaction (PCR) investigations of primary cells and immortalized cell lines indicate that TLRs 1–9 are all expressed by human and mouse lung epithelial cells (3, 5, 58–60).

Toll/Interleukin-1 receptor adaptors (TIR adaptors): host proteins that are selectively recruited to Toll-like receptors and IL-1 and IL-18 receptors upon ligand binding and are required for signal propagation

Lipopolysaccharide (LPS): cell wall component of Gram-negative bacteria, the lipid A portion of which is recognized by TLR4 in association with MD2 and CD14

Cultured respiratory epithelial cells respond to stimulation with TLR agonists by expression of proinflammatory and antimicrobial mediators (60–62b). In vivo, LPS has been administered intranasally and by aerosol to protect against bacterial and fungal lung infections, either by enhancing innate defenses or by attenuating lung injury associated with infection (10, 17, 19). Inhalational or intraperitoneal administration of CpG oligodeoxynucleotides (ODNs) (TLR9 ligand) enhances survival of lung infection by a number of pathogens, including *Mycobacterium avium*, *Klebsiella pneumoniae* and *Burkholderia* species (13, 16, 16a). Treatment of mice with the TLR2/6 agonist MALP-2 induces cytokine production, reduces the pathogen burden, and enhances host survival after challenge with *S. pneumoniae* (18). Mice pretreated with TLR3-stimulating poly I:C or liposomal preparations of TLR9-stimulating CpG ODNs display enhanced survival after challenge with several strains of influenza (57).

Given increasing evidence of cooperative signaling by PRRs (63–65), synergistic combinations of TLR agonists or combinations of TLR agonists and ligands for other PRRs may provide even greater protection than do single ligands alone. Consistent with this option, we have found that certain combinations of synthetic TLR agonists greatly outperform individual ligands in terms of both induced pathogen killing and enhanced host survival (S.E. Evans, M.J. Tuvim & B.F. Dickey, unpublished results). Similarly, by exposing mice to multiple TLR ligands in an aerosolized bacterial lysate, we have found robust survival benefits across a broad spectrum of pathogens (9, 19, 20). The importance of TLRs in general and MyD88 in particular to this response is demonstrated by the complete loss of protection when the aerosolized bacterial lysate is delivered to MyD88-deficient mice, but not when the lysate is delivered to Trif-deficient mice (S.E. Evans & B.F. Dickey, unpublished results). The importance of epithelial MyD88 is indicated by the insensitivity of MyD88-deficient mice transplanted with wild-type bone marrow to intranasal LPS (53, 66) and by their impaired cytokine production and decreased survival when challenged with *P. aeruginosa* in the lungs (23).

Interleukin (IL): a widely expressed and highly variable group of cytokine signaling molecules involved in both innate and adaptive immune responses

NOD-like receptors. The NOD-like receptor (NLR) family is defined by proteins that share a C-terminal LRR domain that interacts with PAMPs, a central nucleotide oligomerization domain (NOD), and one of three N-terminal signaling domains (67). Humans express at least 23 of these proteins, with most apparently restricted to leukocytes, but the best-studied NLRs, NOD1 and NOD2, are expressed by lung epithelial cells (62, 67). Unlike membrane-associated TLRs, NLRs are cytosolic in distribution. NOD1 recognizes γ-D-glutamyl-*meso*-diaminopimelic acid present in the peptidoglycan of Gram-negative and some Gram-positive bacteria (67), whereas NOD2 binds the muramyl dipeptide universally present in bacterial peptidoglycan (68). Activation of signaling via NOD1 or NOD2 results in mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB)-dependent production of proinflammatory mediators, although the details of this cascade are less well characterized than for the TLRs. Mice deficient in these NLRs display increased susceptibility to gastrointestinal bacterial infections, including those caused by *Helicobacter pylori* and *Listeria monocytogenes* (69–71). In the lungs, NLRs are critical to the host response to *S. pneumoniae*, *P. aeruginosa*, *Moraxella catarrhalis*, *Chlamydophila pneumoniae*, and *Mycobacterium tuberculosis* (5, 72–74).

Another important subfamily of NLRs comprises those that induce activation of the inflammasome, a molecular complex that activates caspases to convert prointerleukin (pro-IL)-1β and pro-IL-18 (and possibly pro-IL-33) into their mature forms. The NLRs known to contribute to this response are NALP1, NALP2, NALP3, Ipaf, and NAIP (75). These proteins primarily recognize danger signals, including host inflammatory mediators and crystals, but also detect products of microbial pathogens. For example, the NALP3 inflammasome can be activated by MDP, viral DNA, and bacterial

Complement: a system of more than 30 proteins activated by three pathways (classical, alternative, lectin) that permeabilize pathogens with the membrane attack complex, opsonize microbes, and release fragments with signaling properties such as the anaphylotoxins C3a and C5a

toxins (75, 75a), whereas Ipaf appears to sense bacterial flagellin (76). NALP3 inflammasome activation in lung epithelial cells is required for an effective innate immune response to influenza virus infection in mice (75a–75c). The observation that interleukin (IL)-1β mRNA increases in the lungs of mice by almost 100-fold after treatment with an aerosolized bacterial lysate to induce resistance suggests a role for inflammasome activation (9), and NLRs demonstrate synergistic signaling with TLRs (69).

RIG-I-like receptors. The RIG-I-like receptors (RLRs) are cytosolic PRRs that have been demonstrated to be involved in TLR-independent sensing of viruses and the associated production of type I interferons (77). The RLR family is presently composed of two members: retinoic acid–induced gene-1 (RIG-I) and melanoma differentiation–associated gene 5 (MDA5). RIG-I detects noncapped 5′-triphosphate RNA (78) and appears essential to detecting ssRNA viruses (77). RIG-I-deficient mice demonstrate impaired antiviral responses and increased susceptibility to paramyxoviruses, influenza, varicella, and Japanese encephalitis virus (79). The primary ligand for MDA5 is dsRNA (78), and recent work has shown that MDA5 can detect poly I:C in a TLR-independent manner (79). MDA5 deficiency increases susceptibility to several picornaviruses (79). Consequently, the therapeutic activation of these RLRs may promote antiviral defense, although their role in induced epithelial resistance has not been addressed.

Additional innate receptors. Besides these innate immune receptor families, additional cellular products participate in microbial recognition. For example, the glycosphingolipid lactosylceramide is found on the apical surface of lung epithelial cells and detects fungal β-glucans (80, 81). Other PRRs, such as class A scavenger receptors (e.g., MARCO and SR-AI/II), appear to participate in lung defense, but their expression and function in lung epithelium are unresolved.

Indirect Sensing by Host Signaling

Besides receptors for microbial products, epithelial cells possess mechanisms for detecting the presence of microbes indirectly by sensing the release of molecular constituents from injured neighboring cells (e.g., ATP, adenosine, urate, HMGB1), the activation of extracellular fluid phase proteins (e.g., complement and coagulation cascades), the degradation of extracellular matrix macromolecules (e.g., hyaluronan, elastin), and the secretion of inflammatory signals from leukocytes and other parenchymal cells (e.g., cytokines, eicosanoids).

Host cell products. In normal lung function, ATP is released by mechanical stretch into lung lining fluid, where it performs important homeostatic functions such as regulation of mucin secretion, surface liquid depth, and ciliary beat frequency (82). However, ATP can also be released in large quantities by cell injury, and in this setting ATP and its metabolites are powerful mediators of inflammation. In the gut, ATP that is released mostly from microbes drives T_H17 differentiation in the lamina propria (83), and in the lung, ATP activates dendritic cells in asthmatic airway inflammation (84). Whether epithelial resistance is induced by ATP together with leukocyte recruitment and activation is not known.

Adenosine, generated from ATP or released from cells directly, is an important mediator of inflammation in the lungs as indicated by the lethal pulmonary phenotype of adenosine deaminase null mice and the dependency of allergic and other airway inflammatory disorders upon the presence of adenosine (85). Adenosine signals through four G protein–coupled receptors that have both proinflammatory and anti-inflammatory properties, are expressed on airway epithelial cells as well as immune cells, and interact with PRR signaling in activating leukocytes (85, 86). Adenosine signaling modulates survival in mouse models of bacterial infection, although whether this is through induced resistance or attenuation of excessive inflammation in sepsis is not clear (86). Urate and

calcium pyrophosphate are products of purine metabolism that activate inflammation through the NALP3 inflammasome. NALP3 activation is important for resistance of mice to fungal infection (87), and urate contributes to inflammation during bleomycin-induced lung injury through NALP3 (88), but whether urate contributes to lung resistance to microbial infection is unknown.

Besides the purines, multiple cellular macromolecules termed alarmins can signal to neighboring cells when released during injury (89). These include heat shock proteins, the chromatin-associated protein HMGB1, members of the calcium-binding S100 family, and antimicrobial peptides such as cathelicidin. These have been examined mostly for inflammatory rather than resistance properties, and data on their functions in epithelial cells are scant.

Proteolytic cascades (complement, coagulation, kinins). Besides effector functions in microbial lysis and opsonization (see below), complement promotes resistance by signaling to leukocytes and parenchymal cells. Airway epithelial cells express receptors for the C3a and C5a anaphylatoxins (90), and stimulation by C3a is required for robust epithelial mucous metaplasia in allergic airway inflammation (91, 92). Allergic inflammation is thought to represent an antiparasitic defense (32), and whether C3a or C5a signaling to epithelial cells is similarly required for full induction of antimicrobial resistance is not yet known. The coagulation cascade and platelets are also activated during infection and interact bidirectionally with the complement system (93, 94). Research on interactions of the coagulation system with lung epithelial cells has focused primarily on mesothelial cells of the pleural space in fibrinolysis, permeability changes of the alveolar epithelium in diffuse lung injury, and occlusion of distal airways in asthma (95–97), although the role of the coagulation system in inducible resistance is mostly unknown. The kallikrein-kinin cascade is coactivated with the complement and coagulation cascades, generating potent inflammatory mediators whose role in inducible resistance is yet unknown and activating cathelicidin (98), which likely participates in inducible resistance (see below).

Extracellular matrix products. The hydrolysis of extracellular matrix components releases fragments with inflammatory signaling properties. Prominent among these in the lungs are the glycosaminoglycan hyaluronan, which releases fragments that activate TLR4 (99), and fibronectin, which binds latent transforming growth factor-β (TGF-β) (100), although their roles in microbial resistance within the lungs are not known. In addition to signaling themselves, matrix macromolecules provide nucleation sites for the growth of urate and calcium pyrophosphate crystals (see above), and they are subject to nonenzymatic glycation that is recognized by RAGE (receptor for advanced glycation end products), which has inflammatory activity both alone and in partnership with TLRs (101).

Cytokines and other inflammatory mediators. Cytokines are generated to some degree by all mammalian cells, but most prominently by leukocytes. A subset of leukocyte-generated cytokines induces resistance of epithelial cells to infection (102). First described among these were the type I interferons, which were found in the 1950s to induce epithelial resistance to viral infection (103, 104). In the 1960s, type II interferon (IFN-γ) was discovered, and more recently type III interferons have also been found to be important in epithelial resistance. Besides viruses, interferons also induce resistance to bacterial and fungal pathogens. In the lungs, resistance to *Francisella tularensis* induced by prior administration of a TLR4 agonist was highly dependent upon IFN-γ (15). Other cytokines that induce resistance in epithelial cells are IL-22 and IL-17. IL-22 is produced both by lymphocytes and by natural killer (NK) cells, with the latter providing an innate source for mucosal immunity (105, 106). IL-22 acts primarily on epithelial cells, markedly increasing the production of antimicrobial proteins (21, 105, 107). Importantly, IL-22 induces microbial

Receptor for advanced glycation end products (RAGE): a multifunctional member of the immunoglobulin superfamily that recognizes several host immunomodulatory proteins, including HMGB1 and S100, as well as host proteins without immunomodulatory activity that become glycated during aging or inflammation

Interferon (IFN): a subset of cytokines that inhibit viral replication within host cells and activate leukocytes. There are three classes: type I (α, β, ω, ε, and κ), type II (γ), and type III (λ1-3, also known as IL-28A/B and IL-29), each with distinct receptors

Transmigration: paracellular migration of leukocytes or pathogens through epithelial or endothelial barriers and associated basement membranes

Anoikis: apoptosis of epithelial cells induced by detachment from the extracellular matrix

killing by mouse airway epithelial cells in vitro, and this response depends to a large degree upon lipocalin-2 (21). IL-17 plays important roles in mucosal defense against extracellular lung pathogens (108, 109) and upregulates the production of antimicrobial proteins by lung epithelial cells (107, 110). IL-22 and IL-17 can be coexpressed and can cooperatively enhance the expression of antimicrobial proteins and cytokines by keratinocytes and airway epithelial cells (21, 107). Many other signaling molecules such as eicosanoids, biogenic amines, and proteases also mediate inflammatory signaling, but their roles in induced resistance of epithelial cells are less well defined than those of cytokines.

EPITHELIAL EFFECTOR MECHANISMS OF INDUCIBLE RESISTANCE

Resistance of a multicellular eukaryotic host to infection can be due to either prevention of microbial entry or induction of microbial killing. Epithelial cells participate in both effector mechanisms.

Barrier Function

Epithelia throughout the body serve as mechanical barriers to microbial entry. However, microbes have developed numerous strategies for crossing this barrier by passing between cells, entering and passing through cells, and simply killing cells to eliminate the barrier. Conversely, because it is composed of living cells, the epithelial barrier is capable of plasticity in its ability to resist microbial penetration. Indeed, epithelial barrier functions are modulated both by pathogens and as part of the host response, presenting a dynamic situation during infection. Several recent reviews have discussed the different strategies utilized by microbial pathogens to breach epithelial barriers (98, 111–114). Here we focus on inducible barrier function.

Paracellular transit. Cell-cell junctions and cell-matrix adhesions are critical structures for maintaining the epithelial barrier. It is not surprising that these structures as well as the associated adaptors and regulators are among the most commonly targeted host factors by microbial pathogens to either disrupt the barrier or gain entry into the host cells. For example, transmigration of leukocytes to mucosal surfaces in response to microbial pathogens requires reorganization of the epithelial junctions. In the lungs, this process involves TLR2 signaling, resulting in calcium fluxes that activate calpains, which are cysteine proteases that cleave epithelial junctional proteins (115). Conversely, migration of *S. pneumoniae* and *H. influenzae* across polarized respiratory epithelial cells was shown to be accompanied by disruption of the epithelial barrier mediated by TLR2, p38 MAPK, and TGF-β signaling (116). Whether these two phenomena are connected is not known, although it seems that the immune signaling activated by these pathogens promotes their migration across the epithelial barrier at the same time that it facilitates leukocyte transmigration. In intestinal epithelial cells, TLR2 signaling protects tight junction assembly and gap junction intercellular communication against damage associated with inflammation (117–119).

Transcellular transit. Pathogens at a variety of mucosal surfaces enter epithelial cells to invade underlying tissues (22, 98, 112). Epithelial exfoliation counteracts this pathogenic mechanism. In the distal mammalian gut, which is continuously exposed to microbes (**Figure 2*a***), the epithelium is short-lived (turnover time 5 days), and anoikis likely plays a role in preventing penetration by bacteria that have entered epithelial cells and is antagonized by pathogens (120). In the lungs, which are only intermittently exposed to microbes (**Figure 2*b***), the epithelium is long-lived (turnover time 180 days), and epithelial shedding occurs only during infection or injury (121). In the bladder, uropathogenic *E. coli* invade epithelial cells and establish intracellular reservoirs that serve as sources for recurrent infections. Normal turnover of bladder epithelial cells is slow

(~40 weeks), and stimulating epithelial turnover with protamine sulfate can eradicate infection (122). Furthermore, TLR4 signaling activated by bacterial LPS suppresses the invasion of uropathogenic type 1 fimbriated *E. coli* and *K. pneumoniae* into bladder epithelial cells (123), reducing penetration of the epithelium by these pathogens. In the lungs, *B. anthracis* presents an interesting case. Spores of *B. anthracis* are capable of crossing the lung epithelium by transcytosis without causing apparent disruption to the epithelium or eliciting a strong inflammatory response (124, 125). This capability may be a reason for the lack of symptoms during early stages of inhalational anthrax.

Epithelial injury. Lung pathogens release a wide array of toxins and exoenzymes capable of killing lung epithelial cells and allowing microbial penetration (22, 98, 112). Moreover, viral injury of lung epithelium allows bacterial penetration, which appears to be an important cause of mortality (126). Innate immune stimulation activates NF-κB and kinase cascades that are antiapoptotic and proliferative (3, 22), partially counteracting toxic effects of pathogens. When breaches in the airway epithelium do occur, the growth factor heregulin, which is normally sequestered on the apical surface, gains access to its receptors on the basolateral surface of the remaining cells, initiating a tissue repair response that includes changes in cell shape to rapidly cover the surface, proliferation to reestablish cell number, and promotion of antimicrobial defenses (127).

In summary, the strategies utilized by microbial pathogens to breach epithelial barriers are varied, with modulation of epithelial barrier functions having effects on both the capacity of the host to clear microbes and the ability of pathogens to invade the host.

Microbial Killing

Another important mechanism by which the lung epithelium promotes host survival is through pathogen killing. One means to achieve this is through the engagement of professional immune cells. In response to infection or injury, epithelial cells elaborate abundant proinflammatory and leukocyte chemotactic cytokines, including tumor necrosis factor (TNF), IL-1β, IL-6, IL-8 (or its murine orthologs), granulocyte monocyte colony–stimulating factor (GM-CSF), CXCL5, and leukotrienes, to recruit neutrophils, monocytes, macrophages, and NK cells (128–130). Epithelial cells also express inducible adhesion factors that facilitate leukocyte influx (34, 129).

The epithelium also sculpts the nature of the adaptive immune response. For example, following exposure to certain viruses, helminthes, allergens, and TLR agonists, the lung epithelium produces thymic stromal lymphopoietin (TSLP), IL-25 (also known as IL-17E), GM-CSF, IL-1β, IL-25, and IL-33, promoting selective dendritic cell recruitment and T_H2 deviation of the adaptive immune response (130, 131).

Besides recruiting leukocytes, epithelial cells secrete microbicidal products into the airway lining fluid, and secretion of these products can be increased in response to infection or therapeutic stimulation. These epithelial effectors limit pathogen survival primarily through disruption of their cell walls, through sequestration of iron and other nutrients, and by providing decoy targets for essential microbial metabolic and pathogenic processes (5, 128, 132, 133).

Small antimicrobial peptides. Predominant among the inducible bacteriostatic and bactericidal products of the epithelium are the small cationic antimicrobial peptides, a diverse family of amphipathic, gene-encoded immune effectors. Hundreds of these peptides have been identified in eukaryotes, with tremendous species-specific variation, but human lung epithelium primarily expresses members of just two groups: defensins and cathelicidins (5, 134–140).

Defensins are defined by unique cysteine motifs resulting in three disulfide bonds and

Transcytosis: mechanism of transport across polarized epithelial cells involving endocytosis of extracellular macromolecules or particles on one surface, transcellular vesicle trafficking, and exocytosis on the other surface

are subdivided into categories based on tertiary structure (α, β, and θ). α- and β-defensins are both expressed by neutrophils, but only β-defensins are expressed by the lung epithelium. Human β-defensin-1 (HBD-1) is constitutively elaborated into the airway lining fluid and is further induced by inflammatory stimuli in mechanically ventilated newborns (141, 142). HBD-2, -3, and -4 do not appear to be expressed at baseline but are induced by epithelial cell exposure to pathogens, TLR agonists, or inflammatory cytokines (140, 141, 143, 144). Mice express numerous β-defensins in the lungs, many of them inducible by bacterial and viral infection, and they demonstrate broad antimicrobial activity, including against influenza virus and fungi (145, 146). In our mouse model of stimulation with an aerosolized NTHi lysate, we observed induced expression of several β-defensins (9).

Cathelicidins, like many antimicrobial effectors, are expressed as propeptides that require cleavage of an N-terminal cathelin domain to gain antimicrobial activity. Humans express only one cathelicidin propeptide (CAMP or hCAP-18), which is cleaved to the active antimicrobial peptide LL-37 (135). Lung epithelial LL-37 elaboration can be stimulated by TLR agonists, and production is at least partially vitamin D dependent (147). In the mouse, lung transcripts roughly double after treatment with NTHi lysate (9). There is abundant evidence that cathelicidins promote pathogen clearance in pneumonia (138, 148), so induced production, activation, and secretion may contribute to induced resistance.

Small cationic antimicrobial peptides exert direct antimicrobial effects on Gram-positive, Gram-negative, fungal, and viral pathogens (140). The mechanisms of pathogen killing remain incompletely elucidated, although most literature suggests that the primary mechanism relates to microbial permeabilization (132, 149). This activity may occur by creation of membrane-spanning pores or by detergent-like disruption of pathogen membranes. In fact, antimicrobial peptides may use different mechanisms on different pathogen membranes. Furthermore, there are antimicrobial peptides that kill bacteria without detectable lysis, apparently breaching the membrane to bind critical metabolic targets (139).

The importance of pathogen killing by small cationic peptides to host defense is demonstrated by the impaired pathogen clearance from the lungs and decreased survival of mutant mice deficient in cathelicidin or defensin when challenged with a number of bacterial species (128, 147, 150). Further substantiating the importance of antimicrobial peptides in lung defense, transgenic overexpression of LL-37 in the airway epithelium of mice enhances protection against bacterial pneumonia and sepsis (151, 152). Many antimicrobial peptides demonstrate pathogen-killing effects against bacteria, fungi, protozoa, and some viruses when tested in vitro (139), and virtually all peptides with a net positive charge and a few hydrophobic residues show some antimicrobial activity in nonphysiologic dilute media (137). However, in vivo testing reveals that mammalian antimicrobial peptides have evolved enhanced activity against the pathogens most often encountered in the niche where they are expressed.

Stimulated epithelial production of antimicrobial peptides is also important to lung defenses through their immunomodulatory properties, complementing the actions of epithelium-derived cytokines. For example, hBD2 is chemotactic for mast cells, whereas hBD3 and -4 recruit monocytes and macrophages (153–156). Similarly, LL-37 recruits neutrophils, monocytes, mast cells, and T cells, but not dendritic cells (157, 158). The antimicrobial peptides also exert indirect chemotactic effects, as they enhance secretion of proinflammatory and chemotactic cytokines from local leukocytes (139, 155). Conversely, as shown in keratinocyte and leukocyte models, LL-37 attenuates TLR4-dependent cytokine secretion, indicating that antimicrobial peptides also play a role in immune regulation.

Large antimicrobial proteins. Besides small cationic antimicrobial peptides, several larger

proteins elaborated by the epithelium promote resistance, as recently reviewed (102). Among these, lysozyme is expressed in the greatest abundance. Lysozyme was among the first antimicrobial effectors identified in pulmonary secretions and hydrolyzes β_{1-4} glycosidic bonds in peptidoglycan, disrupting Gram-positive bacteria (159). Increased lysozyme is measureable in lung lining fluid after treatment of mice with an aerosolized NTHi lysate (19). Similarly, lactoferrin is induced by many infectious and inflammatory stimuli, including NTHi lysate (9, 19). Lactoferrin has been presumed to exert its antimicrobial effects through sequestration of iron from pathogens, although it appears to be directly bactericidal as well (5, 159).

Another group of inducible epithelial antimicrobial products comprises the surfactant collectins. Collectins contain an N-terminal cysteine-rich domain, a collagen domain, a coiled-coil neck domain, and a carbohydrate recognition domain (CRD, or C-type lectin domain) (160). As innate immune molecules, they function as soluble PRRs; surfactant proteins A (SpA) and D (SpD) are the best characterized. The surfactant collectins recognize conserved sugar patterns present on respiratory pathogens. Collectin binding of microbes can result in pathogen opsonization and neutralization through agglutination while modulating dendritic and T cell responses (161, 162). We find SpD greatly increased in lung lining fluid following treatment with NTHi lysate (19). However, in some cases, the binding of collectins to microbes may actually promote adhesion and facilitate infection, as in pneumocystis pneumonia (163). Thus, the therapeutic augmentation of collectins may have some practical limitation.

Lipocalin-2 is an epithelium-expressed protein that binds iron siderophores, is important in defense against *K. pneumoniae* pneumonia (164, 165), and appears to be particularly important to induced resistance. Lipocalin-2 is markedly upregulated by IL-22, and its deficiency greatly reduces in vitro microbial killing by mouse tracheal epithelial cells after stimulation with IL-22 (21). We find increased lipocalin-2 gene expression together with increased protein in lung lavage fluid after stimulation of mouse lungs with aerosolized NTHi lysate (9, 19). S100 protein family members, including calgranulins A and B, are expressed by airway epithelial cells and exert antimicrobial effects on a host of respiratory pathogens in a manner independent of their calcium-binding domains (5, 102). These are also increased by NTHi lysate (9, 19). A family of proteins known as palate-lung-nasal-clone (PLUNC), composed of short (SPLUNC1 and -2) and long (LPLUNC) members, shares features with many of the other antimicrobial effectors. There is evidence these proteins contribute to lung epithelial defenses (166, 167), although we do not find them increased by NTHi lysate aerosol. In addition to its leukocyte chemotactic activity, CCL20 (also known as LARC or MIP-3α) shares structural homology with β-defensins and has intrinsic antibacterial activity against Gram-negative pathogens (168). CCL20 mRNA increases in the lungs by more than 200-fold after NTHi lysate treatment (9). Secretory leukocyte proteinase inhibitor (SLPI) and elafin are constitutively expressed in the airways and are further induced from the epithelium in response to infection. Although they appear to limit host injury following infection through protease inhibition, both also exert direct toxic effects on bacterial species (5), and we observe the induction of SLPI and numerous elafin-related protease inhibitors after treatment with NTHi lysate (9, 19). Complement promotes microbial clearance by inducing lysis through the membrane attack complex and by opsonization as well as by signaling to leukocytes and parenchymal cells (see above). Local production of complement may be particularly important (169), and complement gene expression is increased during induced resistance of the lungs (9, 19). The significance of complement activation is suggested by the numerous mechanisms employed by pathogens to evade its effects (170).

Secretory leukocyte proteinase inhibitor (SLPI): an epithelium-derived protease inhibitor with intrinsic antibacterial activity

Reactive oxygen species. The airway epithelium also generates reactive oxygen species

at bactericidal concentrations in response to infection or therapeutic stimulation (171–173). The hydrogen peroxide source for reactive oxygen species is generated primarily in the airway via the dual NADPH oxidase/peroxidase (Duox) system, with Duox 1 constitutively expressed and Duox 2 induced by inflammatory cytokines and infection (174). The bactericidal effects of hydrogen peroxide are greatly enhanced by the presence of peroxidases, and the airway epithelium produces abundant lactoperoxidase (175, 176). Nitric oxide is also produced both constitutively and inducibly in the lung epithelium and contributes importantly to microbial killing, although the relative importance of production by epithelial cells versus macrophages within the lungs is not known (177).

Just as the respiratory epithelium exhibits synergistic PRR signaling, antimicrobial effector molecules expressed by the lungs display synergistic killing. Subinhibitory concentrations of lysozyme and lactoferrin enhance the killing of bacteria by LL-37 and hBD2 (5, 139); combinations of lactoferrin, SLPI, and lysozyme show synergistic killing (178); and antimicrobial proteins interact with reactive oxygen species, as discussed above.

MICROBIAL EVASION OF EPITHELIAL RESISTANCE

Not surprisingly, in view of the efficacy of induced epithelial resistance in suppressing microbial colonization and invasion, pathogens have evolved a wide variety of mechanisms to subvert induced resistance. Many of the signaling pathways and effector mechanisms described above are targeted by pathogens, and this subject has recently been reviewed (98, 170, 179, 180).

DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

From a diagnostic standpoint, epithelial innate immune mechanisms are increasingly recognized as contributing to inflammatory diseases of the gastrointestinal tract, skin, and airways. The contributions of these mechanisms to resistance to microbial infection are less well understood, although spontaneously occurring mutations in humans dovetail informatively with targeted mutations in mice. For example, deficiency of IFN-γ signaling leads to increased susceptibility to mycobacterial infections of the lungs, whereas deficiencies of type I or type III IFN signaling lead to increased susceptibility to viral infections (181), and deficiency of MyD88 leads to pyogenic bacterial infections (182). The contribution of the epithelium to these susceptibilities is being uncovered by hematopoietic stem cell transplantation in humans and tissue-specific studies in mice (183).

From a therapeutic standpoint, innate immunity is being targeted to attenuate inflammatory diseases, provide adjuvant activity in vaccines, deviate the immune system in allergic diseases, and prevent tumor promotion (184, 185). The lung epithelia of human subjects have not been stimulated therapeutically to prevent or treat infection, although topical TLR7/8 agonists are currently in use for treatment of viral and parasitic infections of the skin and genitourinary tract, and TLR9 agonists are being developed for treatment of hepatitis C (184, 185). The rapid, broad, and high level of resistance that can be transiently induced in the lungs of mice suggests that human populations transiently at high risk of pneumonia may benefit from treatment. Such populations may include (*a*) cancer patients being treated with myeloablative chemotherapy and (*b*) the general population during an epidemic for which a vaccine is not available or during a bioterror attack in which the agent is not known or the population cannot be fully protected with existing therapies (186).

CONCLUSION

The efficacy of the lung epithelium in microbial killing has been neglected relative to its roles in signaling to leukocytes and acting as a mechanical barrier. This neglect is due in part to the requirement for stimulation before the

antimicrobial capabilities of the lung epithelium become apparent and in part to the overshadowing of the lung epithelium by the dazzling array of antimicrobial activities displayed by the wide variety of mammalian leukocytes. A better understanding of inducible lung epithelial innate resistance is likely to lead to insight into the variable individual susceptibility to infection and the ability to manipulate resistance therapeutically.

SUMMARY POINTS

1. Inducible innate immune resistance to microbial infection in the lungs is mediated primarily by epithelial cells.
2. Lung epithelial cells are conditional pathogen-killing machines that require stimulation for efficient effector function.
3. Lung epithelial cells are capable both of directly sensing microbes and of responding to signals from leukocytes and other host cells to increase resistance.
4. Although the surface area of the lungs presents a large target for microbial invasion, lung epithelial cells that are closely apposed to deposited pathogens are ideally positioned for microbial killing when activated.
5. The full diagnostic and therapeutic implications of the inducibility of innate immunity in lung epithelium remain to be determined.

FUTURE ISSUES

1. The necessary and sufficient pathways for epithelial sensing of innate immune stimuli to confer resistance against specific pathogens remain to be fully elucidated.
2. The necessary and sufficient epithelial effector mechanisms in the mediation of resistance to specific pathogens remain to be fully elucidated.
3. The relative importance of direct pathogen sensing by epithelial cells and positive and negative signaling from host cells and extracellular macromolecules in the achievement of homeostasis remains to be determined.
4. Whether stimulation of innate resistance of lung epithelial cells can have clinical therapeutic value remains to be determined.

DISCLOSURE STATEMENT

S.E.E., M.J.T, and B.F.D. own stock in Pulmotect, Inc., which is developing methods for stimulating innate immunity within the lungs to prevent and treat pneumonia. B.F.D. serves on the Board of Pulmotect, Inc. Y.X. does not have any financial interest in the subject matter discussed in the manuscript.

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